ORIGINAL ARTICLE

Evaluation of the E-test for routine testing of the susceptibility of *Streptococcus pneumoniae* to benzylpenicillin, amoxicillin and cefotaxime

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Objective: To study the routine use of the E-test for susceptibility testing of penicillin-resistant *Streptococcus pneumoniae.*

Methods: A multicenter study of penicillin-resistant *S. pneumoniae* (PRSP) was carried out in Brittany, France (10 general hospitals, and two university hospitals including a coordinating center). Each hospital detected PRSP by the oxacillin (5-μg) disk method and determined the MICs of penicillin G, amoxicillin and cefotaxime by the E-test under routine conditions. All the PRSP strains were collected in a coordinating center and the MICs were checked by the agar dilution method. The classifications obtained from the MICs determined by the E-test and by the reference method were compared.

Results: Between 1 July 1993 and 30 June 1994, 128 PRSP strains were collected. Agreement within 1 $\log_2$ dilution was obtained for only 62% of strains with benzylpenicillin, 72.5% with amoxicillin and 76% with cefotaxime. These data are well below published values. In addition, 52% of the strains found to be penicillin-resistant by the reference technique were of intermediate resistance according to the E-test. There were major differences in the quality of the results obtained by the participating laboratories.

Conclusions: There are problems of standardization in the routine use of the E-test. Microbiologists should therefore take particular care when performing the test and when reading the results, and ensure that reference strains are included in the assay.

Key words: E-test, MIC, *Streptococcus pneumoniae,* β-lactam

INTRODUCTION

Pneumococci have become considerably more resistant to benzylpenicillin in recent years [1–5], and 25.1% of the isolates tested in France in 1993 were resistant to this antibiotic [6]. This has led to clinical problems in the treatment of meningitis and acute otitis [7–11]. The degree of resistance demonstrated depends upon the specific β-lactam used, and it is the high resistance of some strains that is mainly responsible for treatment failures [9,12]. In addition, although third-generation cephalosporins may be indicated in the most severe disorders, the development of strains that are highly resistant to these molecules can jeopardize their effectiveness [7,13,14].

It is therefore important to specify the degree of resistance of the strain by determining the minimal inhibitory concentrations (MICs) of individual relevant antibiotics. The technique used should be reliable, easy to perform, rapid and reproducible. Several studies have shown that the E-test (AB Biodisk, Solna, Sweden) fulfills these requirements and appears to be suitable for determining the MICs of β-lactams for *Streptococcus pneumoniae* [15–19]. Hence this technique is now routinely used in many diagnostic laboratories.

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However, the widespread use of the test raises problems of inter-laboratory standardization and requires re-examination of the reliability of the test under these new conditions of use.

This study was therefore carried out to compare the MICs obtained with the E-test used under routine conditions in 12 hospital laboratories in Brittany, France, with those obtained by a reference technique of agar dilution performed in a single coordinating laboratory. The results indicate that the E-test is not as effective under routine conditions as in the controlled trials carried out in reference centers, and that its use may lead to errors of classification.

MATERIALS AND METHODS

Strains of pneumococci with reduced susceptibility to penicillins (penicillin-resistant *Streptococcus pneumoniae*: PRSP) were detected in 12 hospital laboratories in Brittany, two university hospitals (Brest and Rennes) and 10 general hospitals (Auray, Dinan, Fougères, Guingamp, Lannion, Lorient, Paimpol, Quimper, St Brieuc and Vannes) between 1 July 1993 and 30 June 1994. The PRSP strains were screened with a 5-μg oxacillin disk [20]. A diameter of <25 mm was taken to indicate a PRSP isolate. The MICs of benzylpenicillin, amoxicillin and cefotaxime were then determined for each of these strains by the E-test in all the participating laboratories. The manufacturer's instructions are shown in Table 1. The tests were routinely performed in all laboratories, as shown in Table 1.

All the PRSP strains were collected in a coordinating center and the MICs were checked by the reference method of agar dilution [21]. Doubling dilutions were prepared in Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with 5% horseblood (Biomérieux, Marcy l'Etoile, France). The inoculum was prepared from an 18-h culture resuspended in brain–heart infusion broth (Biomérieux, Marcy l'Etoile, France) supplemented with ascites fluid (Pasteur, Marnes-la-Coquette, France) and grown at 37 °C under agitation for 3 h to a 0.5 McFarland turbidity standard. The resulting suspension was deposited with a Steers plater, depositing 1 μL containing approximately 10^4 colony-forming units. The plates were incubated at 37 °C in 5% CO_2 for 18 h. A set of five strains of known MIC, provided by the Centre National de Reference des Pneumocoques (CNRP), was included in the tests.

The strains were classified as susceptible, intermediate and resistant according to the Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM) [22]. Those strains having MICs ≤0.06 μg/mL for benzylpenicillin and amoxicillin were considered to be susceptible, those with MICs 0.1–1 μg/mL intermediate, and those MICs ≥2 μg/mL resistant. The equivalent MICs for cefotaxime were ≤0.5 μg/mL (susceptible), 1–2 μg/mL (intermediate) and >2 μg/mL (resistant). The classifications obtained from the MIC determined by the E-test and by the reference technique were compared. Since the E-test has half instead of full two-fold dilutions, MICs obtained with the E-test were rounded to the next higher $\log_2$ dilution if the MIC fell between the standard two-fold increments of the reference method.

Additionally, in order to verify that the discrepancies resulted from inter-center variations, the MICs of 10 selected strains, showing a difference by the two techniques ≥ ±2 $\log_2$ dilutions for benzylpenicillin, were checked by the CNRP by the reference method. The E-tests for benzylpenicillin were also checked by the coordinating center for 33 strains taken at random

Table 1 Etest conditions prescribed by the manufacturer and used in the participating centers

Center	Medium	Atmosphere[a]	Inoculum[b]	Inoculation
Manufacturer	MH[c] + 5% horse or sheep blood	CO_2	0.5	Swabbing
A	MH/Sheep	CO_2	0.5	Flooding
B	MH/Horse	Aerobic	0.5	Swabbing
C	MH/Horse	CO_2	0.5	Swabbing
D	MH/Sheep	CO_2	1	Flooding
E	MH/Sheep	CO_2	0.5	Swabbing
F	MH/Horse	Anaerobic	1	Flooding
G	MH/Horse	CO_2	0.5	Flooding
H	MH/Sheep	CO_2	0.5	Swabbing

[a]Incubated at 37 °C for 18 h for all participating centers.
[b]Expressed in McFarland turbidity.
[c]MH: Mueller-Hinton agar.

from those showing a difference between the E-test and the reference test of $\geq \pm 1$ $\log_2$ dilution.

RESULTS

In total, 128 PRSP strains were sent to the coordinating center between 1 July 1993 and 30 June 1994 by eight of the 12 participating hospitals. The MICs of benzylpenicillin for 121 strains, of amoxicillin for 120 strains and of cefotaxime for 119 strains were compared by the two methods. The MICs obtained by the two methods were used to classify the strains into susceptible, intermediate and resistant according to the CASFM criteria (Table 2). The MICs obtained by the E-test were compared to those obtained by the reference test (Table 3). The MICs were in agreement within ± 1 $\log_2$ dilution for benzylpenicillin in 75/121 strains (62%), in 87/120 strains (72.5%) for amoxicillin, and in 90/119 strains (76%) for cefotaxime. Statistically significant differences in the level of discrepancy between the two techniques were shown among the individual centers ($p < 10^{-3}$) (Table 4). For example, the MICs obtained in center D were in agreement for penicillin in 100% of strains, whereas the percentage agreement was 20% in center C.

These differences resulted in many strains being classified as susceptible, intermediate or resistant differently, depending on the method used to measure the MIC. The classification based on the MIC obtained by the E-test was in agreement with that based on the MIC obtained by the reference test for only 66/121 strains (54.5%) tested for benzylpenicillin. The agreements were a little better for amoxicillin (62% of strains) and cefotaxime (68%). Most of the minor errors for benzylpenicillin and amoxicillin occurred around the breakpoint separating intermediate and resistant strains. Because the MICs for cefotaxime were somewhat lower, these minor errors occurred around the threshold between intermediate and susceptible strains.

Table 2 Susceptibility determined by the Etest and agar dilution methods

E-test result (no. of isolates tested)	Number of isolates assayed by agar dilution as: Susceptible	Intermediate	Resistant
Benzylpenicillin (121)			
Susceptible	0	0	0
Intermediate	2	18	52
Resistant	0	1	48
Amoxicillin (120)			
Susceptible	11	1	0
Intermediate	2	47	38
Resistant	0	4	17
Cefotaxime (119)			
Susceptible	42	25	0
Intermediate	9	39	0
Resistant	0	4	0

Table 3 Comparison of MICs determined by the Etest and agar dilution

Antimicrobial agent	Number of isolates with the following differences in MIC:[a] <−2	−2	−1	0	+1	+2	>+2
Benzylpenicillin (121 strains)	17	26	32	36	7	2	1
Amoxicillin (120 strains)	11	21	39	34	14	0	1
Cefotaxime (119 strains)	4	15	32	31	27	9	1

[a]Zero indicates number of isolates for which MICs are identical; −1 and +1 indicate ± 1 $\log_2$ dilution difference, etc.

Table 4 Agreement between benzylpenicillin MICs determined by Etest and the reference method, for the eight participating centers

Center	Number of isolates with Etest results showing indicated number of doubling dilutions either above or below the reference method MIC value <−2	−2	−1	0	1	2	>+2	Percentage agreement (%)[a]
A	3	1	8	23	3	–	–	89.5
B	1	–	–	–	–	–	–	NA[b]
C	6	2	2	–	–	–	–	20
D	–	–	1	3	1	–	–	100
E	1	3	9	6	1	–	1	76
F	5	8	3	1	–	–	–	23.5
G	–	10	3	1	–	1	–	27
H	1	2	6	2	2	1	–	71.5

[a]Agreement: % of strains with a difference in MICs $\leq \pm 1$ $\log_2$.
[b]NA: not applicable because not enough strains.

The MICs checked by the CNRP for the 10 strains for which the difference between the two techniques was $\geq \pm 2$ $\log_2$ dilutions for benzylpenicillin were all within ± 1 $\log_2$ dilution of the MICs determined by the reference method by the coordinating center, for all three antibiotics tested (data not shown). The E-tests for benzylpenicillin were checked by the coordinating center on 33 strains having a difference between the E-test and reference method values of $\geq \pm 1$ $\log_2$ dilution. They were within ± 1 $\log_2$ dilution of the MICs obtained by the reference technique (data not shown).

DISCUSSION

The development of PRSP and the therapeutic problems that have been reported due to these strains make it imperative to carry out a precise in vitro study of their sensitivity to β-lactam agents. This work was done using the E-test, which provides overnight results and which is easier to perform than agar dilution. But this test must be performed with great care and the results carefully read to avoid the errors identified in this study.

Reduced susceptibility to penicillin is presently detected using disks loaded with 1 μg or 5 μg oxacillin. This is an inexpensive, simple method, but it only provides qualitative results that cannot be used to distinguish between resistant strains and intermediate strains. However, this level of resistance must be known, because treatment failures have been linked to it [9,12,23]. Neither can the activity of benzylpenicillin be used to predict the activities of other β-lactam agents. While all β-lactam agents have reduced activity against PRSP strains, some molecules are relatively more active [24], and cephalosporin disks cannot be used to reliably detect resistance to these molecules [25]. Lastly, some reports indicate that oxacillin disks can indicate false resistance [26,27], and strains resistant to oxacillin but susceptible to benzylpenicillin have recently been found [28,29]. Only two of the 121 strains tested for susceptibility to benzylpenicillin in the present study showed reduced susceptibility by the oxacillin disk test but were susceptible to benzylpenicillin in the reference test.

It is therefore necessary to determine the MICs of β-lactam agents that are likely to be used clinically. The agar dilution technique is the reference method [21], but it is difficult to set up, time-consuming and expensive, especially when several antibiotics must be tested on a single strain. The broth microdilution method [30] is an alternative, but is equally expensive and delicate. These two techniques are used by reference laboratories and are rarely implemented in routine testing in clinical bacteriology laboratories.

The E-test is a new alternative to the above methods that makes the determination of MIC simple and within the reach of all laboratories. The technique has been shown to be reproducible for pneumococci [19] and to provide reliable MIC data [15,17,18,31]. The present study shows that all the 33 MIC values for benzylpenicillin determined by the E-test and checked by the coordinating center agreed with the reference test value within ± 1 $\log_2$ dilution, despite the fact that 27 of the strains differed by $\geq \pm 2$ $\log_2$ dilutions from the reference value during the initial routine determinations in the 12 hospitals. The checks carried out by the CNRP and the coordinating center show that the E-test provides reliable results. But there were major differences in the results obtained by the participating laboratories. This indicates that there are problems of standardization in running the test. Others have reported similar difficulties. Spicq found that only 77% of isolates had MICs within ± 1 $\log_2$ dilution between E-test and reference test in routine use, compared to 90–100% in controlled trials [32]. The conditions under which the E-test were performed varied greatly, and no conclusion can be drawn as to which technique should be used. Bolmström has shown recently that there were no differences when horse blood or sheep blood were used in the medium, but the use of CO_2 during incubation could produce lower MIC values by a single dilution [33]. Plate reading is particularly critical, as the zone of complete inhibition is difficult to determine in the presence of microcolonies and α-hemolysis. This is probably why the results of centers A and G were very different (Table 4), even though the inocula were the same and the techniques used were identical (Table 1).

Most (32/33) of the MICs determined by the controlled E-test in this study were the same as or one dilution under the values given by the reference test; similar results have been obtained by others [15,16,34], indicating that the E-test underestimates the MICs of β-lactam agents for PRSP. This underestimation of the MIC, especially around the critical threshold, resulted in underestimation of the resistance of several strains (Table 3). Scheel found that 10 of the 16 strains classified as resistant by the reference method were placed in the intermediate category by the E-test [35]. This situation, which could cause problems when selecting a specific antibiotic for treatment, led Scheel to suggest changing the critical values for determining the susceptibility to benzylpenicillin by the E-test [35]. Only clinical studies can indicate the practical importance of these laboratory observations.

The present study is in agreement with published data indicating that the E-test is a good test for rapidly and reliably evaluating the sensitivity of several strains

of *S. pneumoniae* to one or more antibiotics. However, it has revealed problems in the performance of the test and reading the results when used in the routine laboratory. Inoculation, Petri dish drying and incubation are among the steps that must be standardized in all laboratories in order to obtain satisfactory E-tests. The E-test strips must be stored correctly. The system should use horse blood or sheep blood agar and plating with an inoculum with an opacity of 0.5 McFarland by swabbing or, after 100-fold dilution, by flooding. Plates should be incubated with CO_2 and read carefully. Reference strains with different MICs should be tested at the same time. With these precautions, the E-test is a method suitable for determining the resistance to β-lactam agents of strains of pneumococci. This, in turn, will lead to the correct guidance of clinicians in their prescription of an effective antibiotic.

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